The Nonnucleoside Reverse Transcription Inhibitor MIV-160 Delivered from an Intravaginal Ring, But Not from a Carrageenan Gel, Protects Against Simian/Human Immunodeficiency Virus-RT Infection

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Abstract

We previously showed that a carrageenan (CG) gel containing 50 μ M MIV-150 (MIV-150/CG) reduced vaginal simian/human immunodeficiency virus (SHIV)-RT infection of macaques (56%, $p > 0.05$) when administered daily for 2 weeks with the last dose given 8 h before challenge. Additionally, when 100 mg of MIV-150 was loaded into an intravaginal ring (IVR) inserted 24 h before challenge and removed 2 weeks after challenge, $> 80\%$ protection was observed ($p < 0.03$). MIV-160 is a related NNRTI with a similar IC_{50} , greater aqueous solubility, and a shorter synthesis. To objectively compare MIV-160 with MIV-150, herein we evaluated the antiviral effects of unformulated MIV-160 *in vitro* as well as the *in vivo* protection afforded by MIV-160 delivered in CG (MIV-160/CG gel) and in an IVR under regimens used with MIV-150 in earlier studies. Like MIV-150, MIV-160 exhibited potent antiviral activity against SHIV-RT in macaque vaginal explants. However, formulated MIV-160 exhibited divergent effects *in vivo*. The MIV-160/CG gel offered no protection compared to CG alone, whereas the MIV-160 IVRs protected significantly. Importantly, the results of *in vitro* release studies of the MIV-160/CG gel and the MIV-160 IVR suggested that *in vivo* efficacy paralleled the amount of MIV-160 released *in vitro*. Hundreds of micrograms of MIV-160 were released daily from IVRs while undetectable amounts of MIV-160 were released from the CG gel. Our findings highlight the importance of testing different modalities of microbicide delivery to identify the optimal formulation for efficacy *in vivo*.

Introduction

EFFORTS TO DEVELOP TOPICAL microbicides that block HIV infection remain a top priority. The success of a microbicide depends on multiple factors such as the physicochemical properties, mechanism of action, and *in vivo* potency of the anti-HIV agent; the properties of the formulation; formulation acceptability; and user adherence. Disparate findings from three clinical trials of oral/topical tenofovir^{1–3} underscore the need to develop additional microbicides with different active pharmaceutical ingredients (APIs) and modes of delivery to improve efficacy through increased potency, user options, and adherence.

Microbicide formulation plays an important role in efficacy and adherence. The availability of diverse microbicide formulations will allow users flexibility, further improving adherence to a plan for HIV prevention. Most candidate microbicides developed to date are coitus-dependent gels designed to be applied within a certain proximity to sexual intercourse. Though ineffective against HIV, carrageenan (CG) gel was shown to be safe in thousands of women⁴ and is being utilized as a vehicle since it is isoosmolar and possesses favorable rheological properties for the delivery of anti-HIV APIs.^{5,6} Additionally, zinc acetate (ZA) formulated in CG prevents high dose herpes simplex virus 2 (HSV-2) infection in mice⁶ and CG may also be active against human papillomavirus (HPV).^{7–10} Intravaginal rings (IVRs)

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represent a platform for the sustained delivery of microbicides. IVRs made from silicone and ethylene vinyl acetate (EVA) have been used successfully to deliver contraceptives¹¹ and hormone replacement therapy.¹² They are well tolerated by women^{11,13} and have been associated with improved user adherence over gels.¹⁴ Although IVRs represent an important advance in vaginal microbicide design, they are not useful for the prevention of rectal HIV transmission (unless vaginal administration leads to effective levels of APIs in rectal tissues).

We are developing microbicides based on the nonnucleoside reverse transcription inhibitor (NNRTI) MIV-150,^{5,15–17} which is active *in vitro* against diverse HIV-1 isolates at subnanomolar concentrations, including isolates resistant to other NNRTIs (Fernández-Romero, unpublished data). We showed that 50 μ M MIV-150 formulated in CG reduced vaginal simian/human immunodeficiency virus (SHIV)-RT (SIV with the RT from HIV-1_{HXB2}) infection.¹⁵ This activity was increased in the presence of ZA, providing ~90% protection for up to 24 h. Thus, ZA improves antiimmunodeficiency virus activity, as well as broadening activity against HSV-2.⁶ MIV-150 IVRs were also shown to reduce SHIV-RT infection,^{15a} providing the first proof-of-concept data that an NNRTI released from an IVR protects *in vivo*, and setting the stage to advance an IVR loaded with MIV-150 and ZA.

To potentially improve our formulations, we evaluated MIV-160, a next generation product in the urea phenylethylthiourea-thiazole (PETT) family of NNRTIs to which MIV-150 belongs.^{18,19} MIV-160 has a chemical structure and *in vitro* IC₅₀ similar to MIV-150 [both <1 nM (0.37 ng/ml for MIV-150, 0.34 ng/ml for MIV-160)],^{19,20} but has greater affinity for the RT K103N mutant. It is also less hydrophobic than MIV-150, which may improve solubility in genital fluids and increase drug transport and bioavailability *in vivo*¹⁸ as well as improve the steady-state levels achieved by release from an IVR.¹² The synthesis of MIV-160 requires about half the steps needed for MIV-150, which might lead to lower production costs. In this study, we confirmed the antiviral activity of unformulated MIV-160 against SHIV-RT in macaque vaginal tissue *ex vivo*. Then we formulated MIV-160 in a CG gel and EVA IVR and tested them in macaques under the regimens used previously for MIV-150.^{15–15a} The MIV-160 EVA IVRs provided excellent protection from SHIV-RT; the MIV-160/CG gel provided no protection. Critically, *in vitro* release studies demonstrated that EVA IVRs released MIV-160, whereas CG gels did not. These data underscore the importance of API formulation in microbicide development.

Materials and Methods

Macaques

Adult female Chinese and Indian rhesus macaques (*Macaca mulatta*) were housed at the Tulane National Primate Research Center (TNPRC; Covington, LA), which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC #000594). The use of macaques was approved by the Animal Care and Use Committee of the TNPRC (OLAW assurance #A4499-01), and animal care complied with the regulations in the Animal Welfare Act²¹ and the Guide for the Care and Use of Laboratory Animals.²² At the time of challenge, animals ranged in age from 4 to 14 years old and their weights ranged from 4 to 12 kg. Macaques

used in all studies described in this report tested negative for simian type D retroviruses and simian T cell leukemia virus-1, and those used for efficacy studies *in vivo* also tested negative for SIV. Anesthesia was administered prior to and during all procedures, and analgesics were provided afterward as previously described.^{15,16} EDTA blood, vaginal swabs, and tissues were collected and transported overnight from the TNPRC to our laboratories at the Population Council. Peripheral blood mononuclear cells (PBMCs), plasma, and swabs were processed as previously described.¹⁵ Animals that became sick during the study were euthanized using methods consistent with recommendations of the American Veterinary Medical Association (AVMA) Panel on Euthanasia. Details on the animals and their treatment groups are provided in Tables 1 and 2.

Vaginal tissue processing and infection *ex vivo*

Explant studies were conducted using vaginal biopsy tissues from both Chinese and Indian macaques as described for human cervical tissues with modifications.²³ Biopsies were washed twice in phosphate-buffered saline (PBS), cut using accupunch to ~3×3×3-mm pieces, and cultured in 96-well round-bottomed plates in cDMEM [DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 100 μ M nonessential amino acids] (Gibco, Grand Island, NY). Tissues were exposed to unformulated MIV-160 (0.1 or 1 μ M) overnight (~18 h) in the presence of 5 μ g/ml phytohemagglutinin (PHA) (Sigma Aldrich, St. Louis, MO) and 100 U/ml interleukin-2 (IL-2) (NCI BRB Preclinical Repository, Frederick, MD). After incubation, tissues were washed four times in PBS by centrifugation (25°C; 10 min, 200×g) and cultured in the presence of PHA/IL-2.

TABLE 1. INFECTION AND IMMUNE STATUS OF SIMIAN/HUMAN IMMUNODEFICIENCY VIRUS-RT-CHALLENGED MACAQUES AFTER DAILY GEL APPLICATION

Gel	Challenge time post last gel	Animal ID	Infection	Ab ^a
CG	8 h	HM27	+	+
		HM28	+	+
		HM29	+	+
		HM30	+	+
		HM31	–	–
		HM38	–	–
		IC82	+	+
		IC78	–	–
	24 h	IC79	+	+
		IE78	+	+
		IE79	–	–
		IE80	+	+
		IE81	–	–
		IE82	+	+
MIV-160/CG	8 h	IR36	+	+
		IR37	+	+
		IR38	–	–
		IR40	–	–
		IR41	+	+
		IR42	+	+
		IR46	+	+

^aSIV antibody response at 4 weeks postchallenge.

CG, carrageenan; SIV, simian immunodeficiency virus.

TABLE 2. INFECTION AND IMMUNE STATUS OF MACAQUES THAT RECEIVED MIV-160 ETHYLENE VINYL ACETATE INTRAVAGINAL RINGS

IVR	Ring duration in vagina		Animal ID	Infection ^a	Ab ^b
	Prechallenge	Postchallenge			
Placebo	2 weeks	2 weeks	DP69 ^c	+	+
			GR96 ^c	–	–
			BR72 ^c	–	–
			BG88 ^c	+	+
			DJ37 ^c	+	+
	24 h	24 h	DH73 ^c	+	+
			FL97 ^c	+	+
			DG31	+	+
			GN40	–	–
			AN57	+	+
MIV-160	24 h	0 weeks	GK98	+	+
			DH66	–	–
			DJ19	–	–
			DV94	–	–
			FR02	–	–
	24 h	2 weeks	GI45	–	–
			GT30	–	–
			GR96	–	–

^aAnimals with negative plasma viral load were confirmed negative by nested PCR for SIV *gag* DNA in peripheral blood mononuclear cells.

^bPositive SIV Ab response 6 weeks postchallenge.

^cHistorical controls from (Singer *et al.*, Science Translational Medicine, In Press).

IVR, intravaginal ring.

Twenty-four hours after MIV-160 exposure, tissues were infected by immersion in SHIV-RT (10^4 TCID₅₀ per piece) overnight (~18 h) in the presence of IL-2. Then tissues were washed as described above. An aliquot of the fourth wash was kept as the day 0 sample and stored at -20°C . On days 3, 7, 11, and 14 of culture, two-thirds of the medium was replaced with fresh cDMEM containing IL-2. Cell-free tissue culture supernatants were stored at -20°C . Tissues were frozen on day 14 of culture.

Infection in the supernatants and tissues (individual wells) was monitored by the RETRO-TEK SIV p27 Antigen ELISA kit (ZeptoMetrix, Buffalo, NY) and quantitative SIV *gag* polymerase chain reaction (qPCR), respectively. For qPCR, DNA was extracted from tissues using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD). qPCR was performed using the Sybr Green 2x PCR Master Mix (Applied Biosystems, Carlsbad, CA) and the 7000 real time PCR system (Applied Biosystems). Changes in SIV *gag* expression were analyzed by the comparative crossing threshold (C_t) method ($2^{-\Delta\Delta C_t}$ method)²⁴ with Rhesus albumin as an internal control. Primers for SIV were SIV667gag for 5'-GGTTGCACCCCC TATGACAT-3' and SIV731gag Rev for 5'-TGCATAGCCG CTTGATGGT-3'.²⁵ Primers for albumin were RhAlbF or 5'-ATTTTCAGCTTCGCGTCTTTTG-3' and RhAlbRev or 5'-TTCTCGCTTACTGGCGTTTCT-3'.¹⁶

Toxicity assay

Toxicity was measured with a modified version of the previously described assay.²³ Macaque vaginal biopsies were washed, cut, and incubated with MIV-160 overnight, as de-

scribed above. After washing five times in PBS, tissues were cultured in cDMEM containing 0.5 mg/ml of Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma Aldrich) for 2 h at 37°C . After incubation with MTT, each explant was transferred to 1 ml of methanol for 6 h or overnight in the dark. Then 200 μl of the extracted solution was measured spectrophotometrically at 570 nm. Tissue viability was determined by normalizing the optical density at 570 nm (OD₅₇₀) of the formazan product by the dry weight of the explants.

Microbicide gel and IVR formulations

CG (3% w/w, lot numbers 080805A515SR, 090127A515SR, and 090612A515MR) was used as the gel control. MIV-160/CG gel (lot number 100202A825MR) contained 3% (w/w) CG, 50 μM MIV-160 (Medivir AB, Sweden), and 1% DMSO (Sigma Aldrich). Gels were stored at room temperature and used within 28 days of formulation. Gel osmolality, pH, viscosity, MIV-160 content, and anti-HIV activity were verified for each lot prior to *in vivo* use.

Macaque-sized IVRs (2 cm outer diameter, 4 mm cross-section) containing 100 mg MIV-160 (MIV-160 batch number 56983) were prepared from EVA-40 (40% vinyl acetate content) using the solvent casting method. EVA-40 beads (Scientific Polymer Products) and MIV-160 were dissolved in dichloromethane (DCM) (Sigma-Aldrich) with stirring. The solution was poured into a pan, and DCM was evaporated to afford a homogeneous thin film that was frozen, ground into 4×4 -mm fragments, melted at 93°C , and then injected into ring molds at 75 psi. Cooled IVRs were removed from molds and stored in the dark at RT.

MIV-160 *in vitro* release

In vitro release of MIV-160 from the CG gel was performed at 37°C and 75% relative humidity. Then 500 mg of the MIV-160/CG gel was placed in the donor chamber of a 9 mM Franz Cell (type 3 flow porting) manufactured by PermeGear (Hellertown, PA). The donor chamber was separated from the 5-ml receptor chamber by a 1-kDa cutoff membrane that allowed free but not CG-bound MIV-160 to pass through. Aqueous 10 mM sodium acetate buffer pH 4.5 (a buffer in which MIV-160 is soluble) was placed in the receptor chamber and stirred with a magnetic stir bar. After 24 h, the buffer was concentrated, and the MIV-160 content was determined by HPLC [lower limit of quantification (LLQ) 4 ng/ml].

In vitro MIV-160 release from IVRs was evaluated using the nonionic surfactant Solutol (0.05 wt%) in 100 ml 25 mM sodium acetate buffer pH 4.2 (SNaC buffer).²⁶ For extended release studies MIV-160 IVRs were suspended in the SNaC buffer at 37°C with shaking and release medium was replaced daily (except for weekends). MIV-160 content was measured in triplicate at the indicated time points by high-performance liquid chromatography (HPLC) using a 150×4.6 mm, 3 μM , C₁₈ column: injection volume 100 μl , mobile phase 50% acetonitrile and 50% 200 mM ammonium acetate buffer, pH 5.0; flow rate 1 ml/min; wavelength 260 nm; column temperature 35°C .

Antiviral activity assay

The antiviral activity of MIV-160 released from IVRs *in vitro* and *in vivo* was measured in aliquots from the *in vitro* release

studies and in vaginal swabs, respectively. Antiviral activity was evaluated against HIV-1_{ADA-M} [lot # P4189, MOI ~0.001, AIDS Vaccine Program (SAIC-Frederick, National Cancer Institute at Frederick)] using the TZM-bl-based multinuclear-activated galactosidase indicator (MAGI) assay.²⁷ Samples diluted 1:10 were titrated 3-fold for a total of six dilutions and assayed in triplicate. Stock MIV-160 diluted to 3.4–0.014 ng/ml (10–0.04 nM) was used in each experiment to control for IC₅₀ variations. IC₅₀ values were calculated using a dose-response-inhibition analysis and MIV-160 concentrations previously measured in the samples (GraphPad Prism v5.0).

In vivo treatments and virus challenge

For efficacy studies, a single 30-mg intramuscular injection of Depo-Provera (depo) was given to each animal 5 weeks prior to virus challenge in order to thin the vaginal epithelium and provide optimal infection in the control group.^{15,28} Chinese macaques were used for the gel study and Indian macaques for the IVR study. We have previously observed no difference in the infection frequency in Chinese and Indian macaques challenged with SHIV-RT.^{15,17,29} In the gel study (Table 1), 2 ml of the MIV-160/CG gel or the CG vehicle was applied daily for 2 weeks as previously described.¹⁵ Eight hours after the last application, macaques were challenged with 10³ TCID₅₀ SHIV-RT.¹⁵ All CG-treated animals were historical controls from a previous study in which they were challenged with the same virus stock.¹⁵ In the MIV-160 EVA IVR study (Table 2), EVA IVRs containing either 100 mg MIV-160 ($n=7$) or no drug ($n=4$ real time and $n=7$ historical controls, $n=11$ total placebo group) were inserted into the vagina 5 weeks after depo treatment, and 24 h later the animals were challenged with 10³ TCID₅₀ SHIV-RT.¹⁵ IVRs were removed 2 weeks postchallenge. Rings remained in place in all animals for the duration of this study. Combining the ring retention data from this current efficacy study (11/11) and our prior ring efficacy study (19/22) we found that 91% (30/33) of EVA-40 rings remained in place for up to 28 days. Animals in both studies were followed for 20 weeks to observe infection status. All animals challenged after treatment with the MIV-160/CG gel herein were naive to MIV-150, MIV-160, and SHIV-RT at the start of the study. In IVR studies, two animals that received placebo IVRs (DP69 and BG88) and four animals that received MIV-160 IVRs (DH66, DJ19, DV94, and FR02) had received MIV-150 IVRs and another animal (GR96) had received a placebo IVR in a previous efficacy study using this challenge virus (Singer *et al.*, unpublished observations). Of note, DP69 and BG88 both became infected in the current study (Table 2). All animals were exposed to SHIV-RT ≤ 1 time prior to this study.

For antiviral activity studies, animals ($n=6$) were depoted as in efficacy studies and 5 weeks later, MIV-160 EVA IVRs were inserted. Vaginal swabs were collected at baseline as well as 24 h, 72 h, and 2 weeks post-ring insertion. IVRs were removed 2 weeks post-ring insertion.

Virus stock

SHIV-RT for use in explant studies and *in vivo* challenge was generated from the original stock provided by Disa Böttiger (Medivir AB)¹⁷ using PHA-activated macaque PBMCs and titrated before use in CEMx174 cells.¹⁶

Determining SHIV-RT infection

Plasma viral RNA copies were measured by quantitative RT-PCR for SIV *gag* in plasma.¹⁶ Nested PCR for SIV DNA in PBMCs was performed as previously described.^{15,16} SIV-specific antibodies (Abs) were monitored by ELISA 4 weeks postchallenge.^{15,16}

Statistical analyses

Data from the *ex vivo* activity and MTT assays were evaluated using the Kruskal–Wallis test. *In vitro* release data were evaluated with Student's *t*-test. Comparison of the percentage of SHIV-RT-infected animals in the differently treated groups was performed using Fisher's exact test. All statistics were calculated using Graph Pad Prism version 5.02 (GraphPad Software, San Diego, CA). Statistical significance was determined by $p < 0.05$.

Results

MIV-160 protects macaque vaginal explants from SHIV-RT

Prior to evaluating the protective effects of formulated MIV-160 *in vivo*, we tested native MIV-160 for protective activity against SHIV-RT in the macaque vaginal explant model. We exposed vaginal biopsies from five animals to 1 μ M or 0.1 μ M MIV-160 for 18 h and infected the biopsies with 10⁴ TCID₅₀ SHIV-RT 24 h later. Infection in the cultures was monitored for 14 days by p27 ELISA. Pre-exposure with 1 μ M MIV-160 conferred complete protection in tissue from four of five animals (Fig. 1A). In tissue from the fifth animal, low-level infection was detected at day 14 only. Decreasing the preexposure dose of MIV-160 to 0.1 μ M gave complete protection in tissue from only one out of five animals. However, infection in the tissues from the other four animals never reached the levels of the positive control, indicating reduced infection at this concentration. The area under the curve (AUC) of the infection over the entire 14 days highlights the strong protective activity of 1 μ M MIV-160 against SHIV-RT (Fig. 1B). qPCR-based quantification of tissue infection on day 14 substantiated the p27 data (Fig. 1C). The average C_t values for SIV detection in 1 μ M MIV-160-treated tissues were >30 and therefore considered undetectable, indicating the absence of infection. Two of the measured C_t values for SIV detection in the medium control were excluded from the $2^{-\Delta\Delta C_t}$ calculations because they were out of range (>30) and consequently inaccurate. These values were from single pieces of tissue with no detectable infection at day 14, which was also observed by ELISA quantification of the supernatants (Fig. 1A).

To verify that MIV-160 does not cause toxicity, we measured tissue viability with the MTT assay. Explants from six macaques were cultured with or without 1 μ M MIV-160 overnight or with gynol (1:10) included in parallel as a toxicity control. Exposure to 1 μ M MIV-160 resulted in no significant decrease in viability (mean OD₅₇₀/dry weight 199.8 ± 12.42) compared to untreated controls (244.4 ± 12.18). In contrast, exposure to gynol resulted in a significant viability decrease (79.8 ± 4) compared to both MIV-160-treated and untreated groups ($p < 0.05$).

FIG. 1. Preexposure to MIV-160 protects macaque vaginal mucosa from infection with simian/human immunodeficiency virus (SHIV)-RT. Tissue explants (five macaques) were exposed to 0.1 or 1 μ M MIV-160 overnight during PHA/IL-2 activation, and challenged overnight with 10^4 TCID₅₀ SHIV-RT/explant 24 h later. Tissues were cultured for 14 days and monitored for infection. Experiments were performed in triplicate or duplicate, depending on tissue availability. **(A)** Mean $\pm$ SEM pg/ml of p27 from individual donors (animal IDs are indicated above each axis). **(B)** AUC of the five pooled experiments. **(C)** p27 and SIV *gag* quantification via ELISA and qPCR, respectively, in infection controls (medium) and MIV-160-treated samples 14 days after challenge. The average C_t values for SIV detection in 1 μ M MIV-160-treated tissues were >30 (out of range), indicating no infection, and thus represented by zeros.

MIV-160/CG gel does not protect macaques from vaginal SHIV-RT infection

Having shown that MIV-160 was nontoxic and blocked SHIV-RT infection *ex vivo*, we formulated MIV-160 in CG gel and evaluated release *in vitro* and efficacy *in vivo*. Using the Franz cell system, we found that MIV-160 was not released

from the CG gel at detectable levels (LLQ 4 ng/ml). Since *in vitro* release may not accurately predict *in vivo* release and efficacy, and the 50 μ M MIV-150/CG gel reduced infection $>50\%$,¹⁵ we evaluated the efficacy of the MIV-160/CG gel in macaques under the same regimen. Then 50 μ M MIV-160/CG or CG alone was administered to macaques daily for 2 weeks followed by SHIV-RT challenge 8 h after the last gel application (Table 1). In the MIV-160/CG group, five of seven animals became infected (72%) compared to 9 of 14 animals (65%) that received CG (Fig. 2A and B). Plasma viral loads in both the MIV-160/CG and CG groups typically peaked 2 weeks postinfection before declining to a similar set point (Fig. 2A and C). There was no significant difference in viremia between infected animals that received CG or MIV-160/CG gels (Fig. 2C).

MIV-160 EVA IVRs provide significant protection against vaginal SHIV-RT infection

The potent activity of MIV-160 against SHIV-RT in vaginal explant tissues coupled with its lack of efficacy *in vivo* when formulated in CG gel underscored the need to explore an alternative MIV-160 delivery mode. Therefore, we formulated 100 mg MIV-160 in EVA-40 matrix IVRs (MIV-160 is uniformly distributed throughout the IVR) and evaluated *in vitro* MIV-160 release using the SNaC buffer as the release medium.²⁶ Daily release over 28 days ranged from 1,197 to 294 μ g/day, averaging 532 μ g/day with a day 14 median of 407 μ g/day (Fig. 3A). The cumulative amount of MIV-160 released after 28 days was 13,400 μ g (mean of the AUC of the MIV-150 release curves), or 13.4% of the loading dose (Fig. 3B). The cumulative percentage of MIV-160 released over time (Q) exhibited partition-controlled kinetics of $Q \propto \text{time}^{0.89}$ (typical of nonsink conditions³⁰) (Fig. 3B). The IC₅₀ value of MIV-160 released into SNaC buffer on day 1 was 0.51 nM [95% confidence interval (CI) of 0.38–0.68], indicating that ring formulation conditions did not impact MIV-160's activity.

FIG. 2. MIV-160/CG gel does not protect against SHIV-RT infection. **(A)** Plasma viral loads (SIV RNA viral copies/ml) are shown over time for each animal challenged with 10^3 TCID₅₀ SHIV-RT 8 h after receiving daily applications of carrageenan (CG) or 50 μ M MIV-160/CG gels for 2 weeks. The number of animals in each treatment group is indicated. **(B)** The frequency of infection is depicted above each bar as the number of infected animals over the number of challenged animals in each group. **(C)** Mean ($\pm$ SEM) plasma viral load of infected animals from each group.

FIG. 3. MIV-160 is released *in vitro* from MIV-160 ethylene vinyl acetate intravaginal rings (EVA IVRs). **(A)** Daily release of MIV-160 over 28 days from 100 mg MIV-160 EVA-40 IVRs in SNaC buffer. **(B)** The cumulative percentage of MIV-160 released (Q) from the MIV-160 EVA-40 IVRs over 28 days. The mean $\pm$ SD from four identical experiments is shown.

Using a regimen previously shown to be effective with 100 mg MIV-150 EVA-40 IVRs,^{15a} we evaluated the ability of MIV-160 EVA-40 IVRs to protect macaques from SHIV-RT. MIV-160 or placebo IVRs were inserted in the vagina 24h before challenge (5 weeks post-depo), and remained in place for 2 weeks after challenge (Table 2). Zero of seven animals that received MIV-160 EVA-40 IVRs became infected (0%) while 8 of 11 macaques that received placebo EVA-40 IVRs became infected (72%) (Fig. 4A and B).¹⁸ This protection was highly significant compared to placebo ($p < 0.005$). Plasma viremia in infected placebo animals typically peaked 2–3 weeks postinfection before declining to a variable set point (Fig. 4A). Complete protection in MIV-160 IVR animals was

FIG. 4. MIV-160 EVA IVRs fully protect against SHIV-RT infection. **(A)** Plasma viremia (SIV RNA copies/ml) shown over time for each animal challenged with 10^3 TCID₅₀ SHIV-RT 24h after receiving placebo or 100 mg MIV-160 IVRs. IVRs were removed 2 weeks post-ring insertion. **(B)** Infection frequency of the placebo and MIV-160 IVR groups is shown above each bar as the number of infected animals over the number of challenged animals in each group. **(C)** Percent inhibition of HIV infection by vaginal swab fluids from animals that received MIV-160 EVA-40 IVRs ($n=6$; mean $\pm$ SEM).

confirmed by nested PCR for SIV DNA in the PBMCs at weeks 2 and 3 (data not shown), and supported by lack of seroconversion (Table 2). All infected animals seroconverted (Table 2).

Vaginal swab samples collected from six macaques at different times post-ring insertion were examined for antiviral activity. We observed a dilution-dependent inhibition of infection by MIV-160-containing swabs (Fig. 4C). MIV-160 release across animals appeared uniform since there was little variation in the antiviral activity of swabs from different animals. Although we could not quantify the amount of MIV-160 in the swabs, the average swab dilution IC₅₀ (the dilution of the swab that inhibited infection by 50%) for MIV-160-containing swabs (0.005, 95% CI 0.0035–0.0084) was similar to that previously observed for MIV-150-containing swabs (0.008, 95% CI 0.0057–0.011).^{15a} In that study, the MIV-150 swab antiviral activity also correlated with the subnanomolar *in vitro* IC₅₀ described for MIV-150, based on the amount of MIV-150 quantified in the swabs.

Discussion

In this study, we demonstrate that appropriately formulated MIV-160 effectively prevents SHIV-RT infection. After verifying that MIV-160 protected cultured macaque vaginal explant tissue from viral infection, having no impact on tissue viability *in vitro*, we evaluated *in vitro* release and *in vivo* efficacy of two formulations of MIV-160 that we had previously evaluated with the related NNRTI, MIV-150: a CG gel and an EVA-40 IVR. The amount of MIV-160 released *in vitro* from the 50 µM MIV-160/CG gel was below the LLQ, and the gel failed to protect macaques from SHIV-RT infection. We were unable to perform pharmacokinetic (PK) measurements in this study as we have not yet developed a suitably sensitive method for the quantification of MIV-160 in biological specimens. Therefore, it is unclear whether MIV-160 was released from CG *in vivo* and delivered to the tissues. However, based on the efficacy data, in this study *in vitro* release from the CG gel predicted the *in vivo* outcome. In contrast, 100 mg MIV-160 IVRs released substantial amounts (micrograms/ml) of MIV-160 per day (in the SNaC buffer) and completely protected macaques from SHIV-RT (100% compared to placebo). This protection, which was similar to that observed for 100 mg MIV-150 IVRs, also correlated with efficient release of active MIV-160 from the IVRs *in vitro* as was observed for MIV-150.^{15a} While we could not measure the MIV-160 levels released by the IVRs *in vivo*, the fact that the swabs obtained from animals carrying MIV-160 rings possessed antiviral activity comparable to that from animals carrying MIV-150 IVRs suggests that similar drug levels were present. This provides the proof-of-concept that MIV-160 delivered by an IVR can prevent vaginal SHIV-RT infection. Future studies are needed to determine whether an MIV-160 IVR would remain protective if the animals were challenged at later time points after ring insertion (when drug levels would be expected to be lower).

MIV-150 and MIV-160 have structural similarities and are active against SHIV-RT with the same *in vitro* IC₅₀. However, MIV-160 is more water soluble than MIV-150, which could impact its release from the delivery vehicle and transit through mucosal tissue.¹⁸ Our *in vitro* release data indicate that about 2.5× more MIV-160 is released from EVA IVRs

compared to MIV-150 EVA IVRs over 28 days (Singer *et al.*, unpublished observations). The enhanced release could be due to a combination of factors including higher solubility of MIV-160 in the EVA, increased water uptake and formation of aqueous channels in the EVA, and greater solubility of MIV-160 in the release medium. Dapivirine, another NNRTI in development as a microbicide, has enhanced steady-state release from silicone IVRs in the presence of hydrophilic excipients.³¹ A recent study demonstrated for silicone IVRs releasing one of the hydrophilic CCR5 inhibitors Maraviroc or CMPD167 that the APIs moved efficiently out of the vaginal fluid, through the mucosal tissue, and into the circulation,³² while we have shown that hydrophobic MIV-150 released from EVA IVRs reaches sustained concentrations within the vaginal fluids and tissues.^{15a} The structural and solubility differences between MIV-150 and MIV-160 could also impact their release from a CG gel, pointing to the importance of developing a wide array of vehicles for delivering different APIs.³³ The outcome of our *in vivo* studies confirms the importance of choosing a suitable delivery system for each API.^{34,35}

MIV-160 provided significant protection against SHIV-RT infection *in vitro* at 1 μ M. HIV susceptibility studies in cultured human genital mucosa have been incorporated into the framework of microbicide testing because tissue explants more closely mimic the *in vivo* setting than *in vitro* cell-based assays.³⁵ Explant studies explained the toxicity associated with gynol (Nonoxynol-9),^{36,37} which enhanced HIV infection in clinical trials.^{38–40} Tissue models also allow for the study of APIs and formulations on infection within specific sub-compartments (e.g., ectocervix, endocervix, and vagina) as a precedent to related *in vivo* studies. Human explant studies are now recommended for products moving from preclinical into clinical development.⁴¹ Based on the originally designed human nonpolarized culture model,²³ we have established a macaque vaginal explant model of SHIV-RT infection for evaluation of APIs and microbicide formulations^{23,42} (Telshova and Barnable, unpublished observations). Our data indicate that unformulated MIV-160 is a potent NNRTI capable of preventing SHIV-RT infection in macaque vaginal explants. However, we did not assess the activity of the MIV-160/CG gel in explants in this study, which might have predicted the *in vivo* outcome.

APIs that could be prepared in multiple dosage forms would be valuable for letting users choose how to protect themselves, potentially boosting acceptability, adherence, and, consequently, efficacy.⁴³ Currently, the lead microbicide candidates in clinical trials are a tenofovir-based gel² and a dapivirine silicone IVR.¹³ In preclinical studies, the 1% tenofovir gel that went on to show clinical promise (1) significantly protected macaques from a repeated low dose challenge when administered 30 min⁴⁴ or 30 min and 3 days⁴⁵ before SHIV exposure, (2) offered significant protection in macaques against rectal infection with SIV,⁴⁶ and (3) prevented HIV infection in human cervical explants.⁴⁷ Development of a polyurethane tenofovir IVR (that codelivers dapivirine) has begun,⁴⁸ but no macaque studies have been published to date. Notably, the 1% tenofovir gel that was tested clinically has drawbacks: it is hyperosmolar (unlike its HEC placebo control) and causes breaks in the epithelium in ectocervical and colorectal explant cultures, although it does not affect viability in the MTT assay.⁴⁷ However, a lower osmolarity, reduced

glycerin tenofovir gel has been developed.⁴⁹ Data from a Phase 1 clinical trial suggested the gel was safe and acceptable to men and women (McGowan *et al.*, CROI 2012, Paper #34LB, Seattle, Washington). Due to its mechanism of action, tenofovir is also much less potent than NNRTIs,^{18,50} requiring more drug for efficacy and increasing the chances for toxicity. Dapivirine IVRs have been extensively studied for *in vitro* release^{31,51} and PK,^{13,52} and they have entered Phase 3 clinical testing; however, no efficacy studies in macaques have been published. A recent report revealed that cervicovaginal fluids taken from women using the dapivirine IVR exhibited antiviral activity *ex vivo* (Nuttall *et al.*, M2012, Session 11, Sydney Australia). Extensive PK studies of dapivirine gel have been conducted in animal models⁵³ and humans,^{54–56} with efficacy demonstrated in the hu-SCID mouse model when the gel was applied 20 min prior to vaginal cell-associated HIV challenge.⁵⁷

MIV-160 is a potent NNRTI that, when delivered appropriately, can prevent immunodeficiency virus infection in macaques. However, it is not useful when formulated in CG, a gel component that is needed to provide the increased breadth of activity against other STIs. Specifically, ZA's anti-HSV-2 activity is potentiated by CG,⁶ and CG has been shown to have activity against HPV.^{6–10} It is possible that delivering MIV-160 in a different gel (e.g., HEC) would be effective; however, this was not pursued herein due to our desire to develop broad-spectrum microbicides with activity against HIV and other STIs. We have previously demonstrated that MIV-150 IVRs are highly effective,^{15a} and that an MIV-150/ZA/CG gel significantly protects macaques from SHIV-RT¹⁵ and mice from HSV-2.⁶ As a result, we will focus our efforts on gels and IVRs that release MIV-150 and ZA, developing safe and effective formulations that will prevent HIV as well as other STIs. Future studies will also involve testing the lead formulations in the repeated, low-dose challenge macaque model.

Collectively, our data indicate a correlation between *in vitro* API release and *in vivo* efficacy, underscoring the importance of formulation and release, explant, and PK studies in microbicide design.

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Author Disclosure Statement

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